

RayBio[®] Human/Mouse/Rat Ghrelin Enzyme Immunoassay Kit

Catalog #: EIA-GHR, EIAM-GHR, EIAR-GHR

User Manual

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Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

3607 Parkway Lane, Suite 100
Norcross, GA 30092

Tel: 1-888-494-8555 (Toll Free) or 770-729-2992, Fax: 770-206-2393

Web: www.RayBiotech.com, Email: info@raybiotech.com

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Please read the entire manual carefully before starting your experiment

I. Introduction

Obesity, which is characterized by excessive accumulation of adipose tissue in the body, has become one of the greatest public health challenges. Obesity is not only associated with health problems linked to increased weight-dependent pressure overload on lung, joints and bones, but also a important risk factor for life-threatening diseases such as cardiovascular diseases, type 2 diabetes and certain cancers.

Ghrelin is synthesized as a preprohormone, and then proteolytically processed to yield a 28-amino acid peptide. Synthesis of ghrelin occurs predominantly in epithelial cells lining the fundus of the stomach, with smaller amounts produced in the placenta, kidney, pituitary and hypothalamus.

Ghrelin has emerged as the first circulating hunger hormone. Ghrelin increases food intake and thus fat mass by an action exerted at the level of the hypothalamus. They activate cells in the arcuate nucleus that include the orexigenic neuropeptide Y (NPY) neurons. Ghrelin-responsiveness of these neurons is both leptin and insulin sensitive. Ghrelin also activates the mesolimbic cholinergic-dopaminergic reward link, a circuit that communicates the hedonic and reinforcing aspects of natural rewards, such as food.

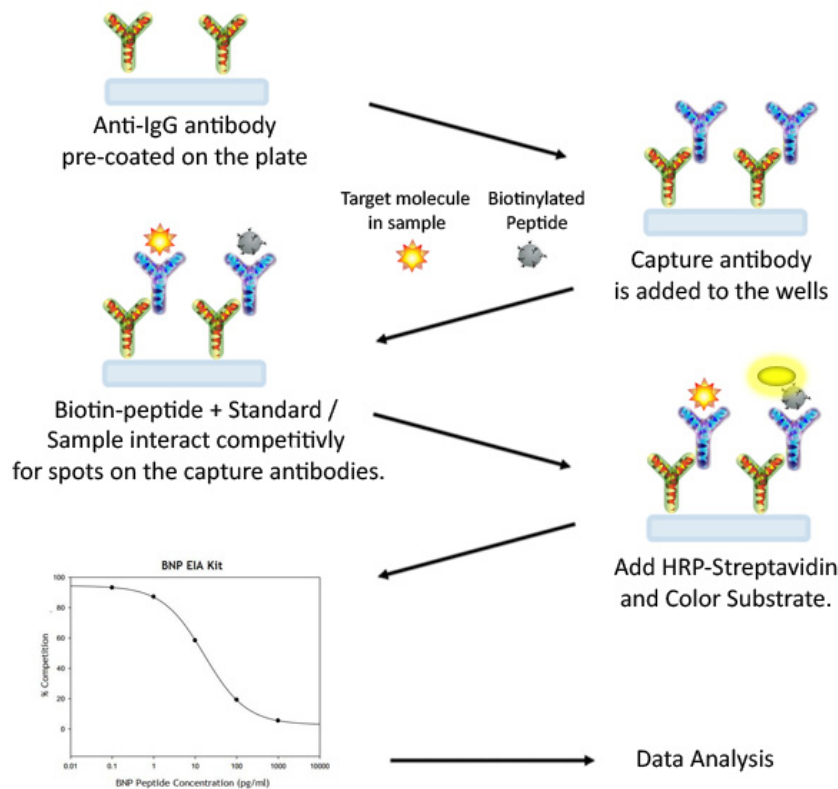
Ghrelin levels in the plasma of obese individuals are lower than those in leaner individuals except in the case of Prader-Willi syndrome-induced obesity. Those suffering from the eating disorder anorexia nervosa have high plasma levels of ghrelin compared to both the constitutionally thin and normal-weight controls. These findings suggest that ghrelin plays a role in both anorexia and obesity. Ghrelin levels are also high in patients who have cancer-induced cachexia.

II. General Description

The RayBio® Ghrelin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Ghrelin peptide based on the competitive enzyme immunoassay principle.

In this assay, a biotinylated Ghrelin peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Ghrelin peptide competes with endogenous (unlabeled) Ghrelin for binding to the anti-Ghrelin antibody. After a wash step, any bound biotinylated Ghrelin then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Ghrelin peptide and inversely proportional to the amount of endogenous Ghrelin in the standard or samples. A standard curve of known concentration of Ghrelin peptide can be established and the concentration of Ghrelin peptide in the samples can be calculated accordingly.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	Size / Description	Storage / Stability After Preparation
Ghrelin Microplate (Item A)	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard Ghrelin Peptide (Item C)	2 vials of Lyophilized Ghrelin Peptide. 1 vial is enough to run each standard in duplicate.	Do not store and reuse
Anti-Ghrelin Polyclonal Antibody (Item N)	2 vials of Lyophilized anti-Ghrelin.	Do not store and reuse
5X Assay Diluent B (Item E)	15 ml of 5X concentrated buffer. Diluent for both standards and samples including serum, plasma, cell culture media or other sample types.	1 month at 4°C
Biotinylated Ghrelin Peptide (Item F)	2 vials of Lyophilized Biotinylated Ghrelin Peptide, 1 vial is enough to assay the whole plate.	Do not store and reuse
HRP-Streptavidin Concentrate (Item G)	600 µl 50X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	1 vial of Lyophilized Positive Control.	Do not store and reuse
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 μ l to 1 ml volumes
3. Adjustable 1-25 ml pipettes for reagent preparation
4. 100 ml and 1 liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

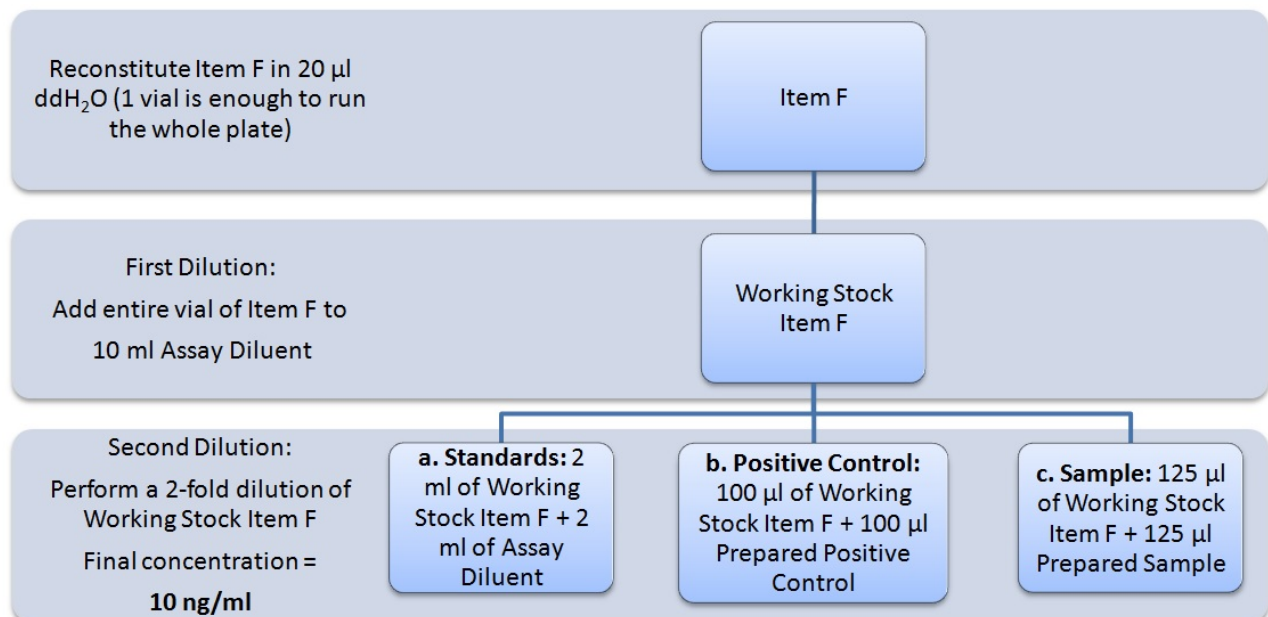
A. Preparation of Plate and Anti-Ghrelin Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-Ghrelin antibody vial (Item N) and reconstitute with 55 μ l of 1X Assay Diluent B to prepare the antibody concentrate. Pipette up and down to mix gently.
5. The antibody concentrate should then be diluted 100-fold with 1X Assay Diluent B. This is your anti-Ghrelin antibody working solution, which will be used in step 2 of Assay Procedure (Section VIII).

Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated Ghrelin (Item F)

5. Briefly centrifuge the vial of Biotinylated Ghrelin (Item F) and reconstitute with 20 μ l of ddH₂O before use.
6. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of 1X Assay Diluent B. This is your Working Stock of Item F. Pipette up and down to mix gently.
*The final concentration of biotinylated Ghrelin will be **20 ng/ml**.*
 - a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of 1X Assay Diluent B. The final concentration of biotinylated Ghrelin will be **10 ng/ml**.
 - b. Second Dilution of Item F for Positive Control: Add 100 μ l of Working Stock Item F to 100 μ l of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated Ghrelin will be **10 ng/ml**.
 - c. Second Dilution of Item F for samples: Add 125 μ l of Working Stock Item F to 125 μ l of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated Ghrelin will be **10 ng/ml**.

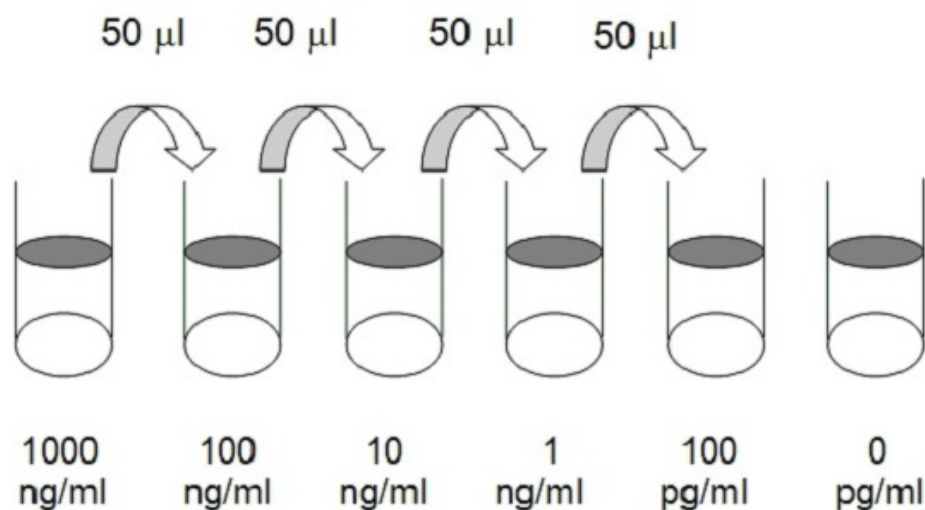


C. Preparation of Standards

7. Label 6 microtubes with the following concentrations: 1000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450 μ l of biotinylated Ghrelin Item F working solution (prepared in step 6a) into each tube, except the 1,000 ng/ml (leave this one empty).

It is very important to make sure the concentration of biotinylated Ghrelin is 10 ng/ml in all standards.

8. Briefly centrifuge the vial of Ghrelin Standard (Item C). Reconstitute with 10 μ l of ddH₂O and briefly vortex if desired. Pipette 8 μ l of Item C and 792 μ l of 10 ng/ml biotinylated Ghrelin working solution (prepared in step 6a) into the tube labeled 1000 ng/ml. Mix thoroughly. This solution serves as the first standard (1,000 ng/ml Ghrelin standard, 10 ng/ml biotinylated Ghrelin).
9. To make the 100 ng/ml standard, pipette 50 μ l of the 1000 ng/ml Ghrelin standard into the tube labeled 100 ng/ml. Mix thoroughly.
10. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 μ l of biotinylated Ghrelin and 50 μ l of the prior concentration until the 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

11. Briefly centrifuge the Positive Control vial (Item M) and reconstitute with 100 μ l of ddH₂O.
12. Refer to step 6b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated Ghrelin should still be 10 ng/ml.

The Positive Control is a cell culture media sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated Ghrelin is 10 ng/ml.

E. Sample Preparation

13. If you wish to perform a 2-fold dilution of your sample, proceed to step 6c. If you wish to perform a higher dilution of your sample, dilute your sample with 1X Assay Diluent B before performing step 6c.
EXAMPLE (to make a 4-fold dilution of sample):
 - a. Dilute sample 2-fold (62.5 μ l of sample + 62.5 μ l of 1X Assay Diluent B.).
 - b. Perform step 6c (125 μ l of working solution Item F + 125 μ l of sample prepared above).

The total volume is 250 μ l, enough for duplicate wells on the microplate.

It is very important to make sure the final concentration of the biotinylated Ghrelin is **10 ng/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human=4X
Mouse=4X Rat=2X.

If you have any questions regarding the recommended dilutions you may contact technical support at 888-494-8555 or techsupport@raybiotech.com.

F. Preparation of Wash Buffer and HRP

14. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.
15. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
16. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use.
17. Dilute the HRP-Streptavidin concentrate 50-fold with 1X Assay Diluent B.

VIII. Assay Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.
2. Add 100 μ l of Anti-Ghrelin Antibody (Item N) (See Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1X Wash Solution Buffer (200-300 μ l each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ l of each standard (see Reagent Preparation Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) in appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) overnight or at 4°C.
5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 μ l of prepared HRP-Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking. It is recommended that incubation time should not be shorter or longer than 45 minutes.
7. Discard the solution and wash 4 times as directed in Step 3.
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 μ l anti-Ghrelin to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.
3. Add 100 μ l standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.
4. Add 100 μ l prepared Streptavidin solution. Incubate 45 minutes at room temperature.
5. Add 100 μ l TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 μ l Stop Solution to each well. Read at 450 nm immediately.

X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

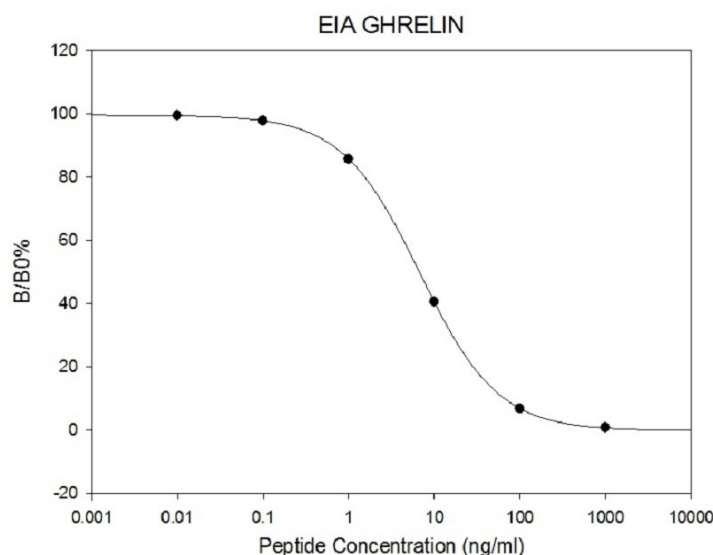
Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where

B = OD of sample or standard and

B_0 = OD of zero standard (total binding)

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The minimum detectable concentrations of Ghrelin is 161 pg/ml or 12.46 pM.

C. Detection Range

0.1-1,000 ng/ml

D. Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard1	Standard1	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard2	Standard2	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard3	Standard3	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard4	Standard4	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard5	Standard5	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32	SA40	SA40

Key:

Blank = Buffer Only

Total Binding = Biotin- Ghrelin only

Standard 1 = 1000 ng/ml

Standard 2 = 100 ng/ml

Standard 3 = 10 ng/ml

Standard 4 = 1 ng/ml

Standard 5 = 100 pg/ml

Pos Control = Biotin with Item M

XI. Specificity

Cross Reactivity: This EIA kit shows no cross-reactivity with any of the cytokines tested: Nesfatin, Angiotensin II, NPY and APC.

XIV. Publications Citing This Product

1. Plum L., et al. The Obesity Susceptibility Gene Carboxypeptidase E Links FoxO1 Signaling in Hypothalamic Pro- α -melanocortin Neurons with Regulation of Food Intake. *Nat Med.* 2009 Oct;15(10):1195-201. doi: 10.1038/nm.2026
Species: Mouse
Sample Type: Serum
2. Fung JN., et al. Expression and In Vitro Functions of the Ghrelin Axis in Endometrial Cancer. *Horm Cancer.* 2010 Oct;1(5):245-55. doi: 10.1007/s12672-010-0047-1.
Species: Human
Sample Type: Cell Lysate
3. Ma X., Zhao Y., Wang Q., Wu L., Wang Z., et al. Plasma Ghrelin Concentrations Are Negatively Correlated With Urine Albumin-to-Creatinine Ratio in Newly Diagnosed Type 2 Diabetes. *The American Journal of Medical Sciences* 2014 June. Epub Ahead of Print. doi: 10.1097/MAJ.0000000000000297
Species: Human
Sample Type: Plasma
4. Han J., Kim H., Lee J., Choi M., Kim Y., Son C. Repeated Sense of Hunger Leads to the Development of Visceral Obesity and Metabolic Syndrome in a Mouse Model . *PLOS One* Published: May 30, 2014. DOI: 10.1371/journal.pone.0098276
Species: Rat
Sample Type: Serum
5. Liu R., Ma D., Li Y., Hu R., Peng Y., Wang Q. The anorexic effect of Ex4/Fc through GLP-1 receptor activation in high-fat diet fed mice. *Acta Biochimica et Biophysica Sinica.* Epub Ahead of Print 2014. DOI: 10.1093/abbs/gmu044.
Species: Mouse
Sample Type: Serum
6. Zhang S., et al. Ghrelin and obestatin plasma levels and ghrelin/obestatin prepropeptide gene polymorphisms in small for gestational age infants. *Journal of International Medical Research* published online 15 September 2014. DOI: 10.1177/0300060514533525
Species: Human
Sample Type: Plasma

For additional publications citing this product please contact technical support at 888-494-8555 or techsupport@raybiotech.com.

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	<ul style="list-style-type: none"> • Inaccurate pipetting • Improper standard dilution 	<ul style="list-style-type: none"> • Check pipettes • Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	<ul style="list-style-type: none"> • Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution 	<ul style="list-style-type: none"> • Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	<ul style="list-style-type: none"> • Inaccurate pipetting • Air bubbles in wells 	<ul style="list-style-type: none"> • Check pipettes • Remove bubbles in wells
High background	<ul style="list-style-type: none"> • Plate is insufficiently washed • Contaminated wash buffer 	<ul style="list-style-type: none"> • Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	<ul style="list-style-type: none"> • Improper storage of the ELISA kit • Stop solution 	<ul style="list-style-type: none"> • Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. • Add stop solution to each well before reading plate

RayBio[®] ELISA Kits

Over 2,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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